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RESEARCH REPORT

In vitro selection and identification of a cold-tolerant variant in pineapple (*Ananas comosus*)

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Abstract

Cold stress is an important factor limiting the growth and distribution of pineapple. Breeding cold-resistant germplasm is an effective way to cope with this problem. In vitro selection for the somaclonal variation using different selection agents has been used for crop improvement in stress tolerance. In this study, the pineapple cold-tolerant variant was developed through in vitro cold selection of somaclonal variations. Low temperature was used as the selected agent, and the extreme lethal condition for the in vitro pineapple cultures was determined to be 0 °C for 72 h. The morphology changes of the in vitro cultures during the cold selection were observed and analyzed. The cold-tolerant variant line was finally obtained through three consecutive selections with cold shock treatments, based on the established high-efficiency culture system for pineapple embryogenic calli. The genetic variations at the molecular level in the cold-tolerant variant were verified by ISSR analysis. The significantly improved cold tolerance in our selected variant was mainly reflected by the higher survival rate, increased proline content, and elevated SOD activity under cold stress compared to these qualities in the control plants. This study demonstrated the feasibility of in vitro selection for cold tolerance in pineapple. The cold-tolerant variant could be valuable for future pineapple breeding programs and for cold tolerance research.

Keywords Pineapple · Cold tolerance · Cold stress · In vitro selection

1 Introduction

Pineapple is the third most important tropical fruit in production and is found in almost all the tropical and subtropical regions of the world (Montero-Calderón and Cerdas-Araya 2012). Pineapple is cold-susceptible, and temperature is one of the most important environmental factors determining its distribution and production (Ogata et al. 2016). Pineapple production is typically hampered by low-temperature injury during the winter period, especially in cool subtropical cultivation regions (Bartholomew et al. 2002). It has been reported that pineapple cannot withstand freezing temperatures and has no root or shoot growth at 7 °C. Prolonged exposure to low temperature could destroy the canopy and lead to the loss of the crop (Py et al. 1987; Lobo and Paull 2017). Therefore, the selection of cold-resistant pineapple genotypes is important. However, conventional breeding efforts are time-consuming and labor-intensive. Moreover, the limited genetic information available for cold-tolerant pineapple has prevented conventional cultivar selection (Bartholomew et al. 2002).

Biotechnology tools like in vitro culture technology offer effective alternatives for the constraints and complications of traditional crop improvement (Maleki et al. 2019). The commonly found somaclonal variations in tissue cultures compensate for the progressively narrow genetic base of many crop plants (Krishna et al. 2016). In vitro selection offers the possibility to select variants from many totipotent cells in a confined space and provides a fast method for developing plants with improved traits, specifically for biotic and abiotic stress resistance (Jain et al. 2013). Several cultivars have been developed through in vitro selection

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of somaclonal variation in different crops for a range of useful traits, such as disease, insect, and herbicide resistance as well as tolerance to cold, heat, metal, and salt stresses (Sengar et al. 2009; Rai et al. 2011; Verma et al. 2013; Ashrafzadeh and Leung 2015; Ge et al. 2015; Shi et al. 2020).

For cold stress, the limiting factor, low temperature, has been successfully exploited in the in vitro selection of cold-tolerant variants from some plant species (Krishna et al. 2016). For example, the somaclonal variants derived from cold-selected calli of seashore paspalum (*Paspalum vaginatum*) exhibited significant improvement in their tolerance to low temperature (Liu et al. 2013). Bertin and Bouharmont (1997) isolated heritable chilling-tolerant somaclonal variants of rice through the selection of embryo-derived calli under low-temperature conditions. The freezing-tolerant variants of St. Augustinegrass, which exhibited a significantly higher survival rate under freezing stress, were selected through four rounds of freezing tests (Li et al. 2010). A cold-tolerant mutant in seashore paspalum was obtained through the combination of induction and selection of enhanced cold tolerance (Shi et al. 2020). Many different variants with improved tolerance to cold stress have been isolated by in vitro selection, which were valuable for the genetic improvement of crop plants.

Many applications of tissue culture for pineapple propagation and improvement, such as micropropagation and regeneration from callus cultures, have already been widely adopted (Bartholomew et al. 2002; Anis and Ahmad 2016; Sanewski 2018; Smith and Botella 2020). A range of somaclonal variants of pineapple have been reported, including variants for leaf colour, leaf shape, and phyllotaxy, and most of the variations were attributed to regeneration of plants from callus cultures (Lobo and Paull 2017). For example, two new pineapple somaclonal variants, P3R5 and Dwarf, derived from the in vitro culture of cv. Red Spanish Pinar, were obtained (Pérez et al. 2012). Somaclonal variation combined with in vitro selection could offer greater potential to isolate pineapple variants with desirable stress tolerance traits.

In this study, in vitro cold selections were performed on cultures derived from the original potential cold-tolerant plant variant. This research aims to establish an effective system for the in vitro selection of pineapple somaclonal variations that are more tolerant to cold stress. The cold-tolerant variant with significantly improved cold tolerance ability was obtained through three rounds of successive cold selection. The cold-tolerant variant could be valuable germplasm for the genetic improvement of pineapple cold tolerance.

2 Materials and methods

2.1 The establishment of a culture system for pineapple in vitro cultures

A potential cold-tolerant pineapple (*Ananas comosus* var. Shenwan) plant variant was found in an orchard in Guangdong Province after a long period of cold weather in 2016. This variant was different from the other pineapple plants in this orchard, which suffered various levels of cold injuries, and grew normally at 4 °C for up to 7 days. Further field observation found that its vegetative progeny also possessed the cold tolerance characteristics. This individual plant was adopted as the maternal plant for subsequent in vitro cultures in our study. Slips were excised from this field-grown maternal plant. The outer leaves were removed, washed thoroughly under running tap water for 15 min, excised with a minimum of the subjacent tissue, and surface-sterilized with 2% NaClO for 10 min followed by repeated washing with sterile distilled water. Then, slips were immersed in 0.1% $HgCl_2$ for 8 min and subsequently rinsed three times with sterile distilled water. The explants were cultured on a callus induction medium (MS medium with 2.0 mg/L BA and 2.5 mg/L NAA) at 25 ± 1 °C under a 16-h photoperiod for 40 days. The induced calli were cut, transferred to solid multiplication medium (MS medium with 3.0 mg/L BA and 2.0 mg/L NAA) for further multiplication, cultured in the dark, and sub-cultured every 30 days. For induction of somatic embryos, MS medium with 2.0 mg/L 2, 4-D and 0.5 mg/L BA was used (Luan et al. 2019). For shoot induction, calli were inoculated on MS medium with 2.0 mg/L BA and 1.0 mg/L NAA. For root induction, regenerated shoots were then transferred to MS medium with 2.0 mg/L IBA.

2.2 Experiments for testing cold tolerance of pineapple in vitro cultures

The well-grown embryogenic calli and regenerated plantlets were selected and inoculated on the pre-cooled solid multiplication medium. Each treatment was replicated in 5 bottles and 10 callus aggregates or plants per bottle. The control cultures were placed at 5, 2, and 0 °C in growth chambers for 12, 24, and 48 h, while the cold-tolerant cultures were treated at 8, 5, 2, 0, and −2 °C for 6, 12, 24, 48, and 72 h. The low-temperature-treated in vitro cultures were further incubated in a 16-h photoperiod at 25 ± 1 °C. The survival rate of the embryogenic calli was calculated as the proportion of the calli with surviving somatic embryos in the total calli aggregates, while the survival rate of the regenerated plantlets was calculated as the proportion of the surviving plantlets in the total cold-treated plantlets.

2.3 TTC method

The viability of embryogenic calli was measured by the TTC method (Steponkus and Lanphear 1967). The embryogenic calli (0.3 g) were suspended in 3 mL of 0.6% TTC (W/V) solution prepared in 50 mM phosphate buffer (pH = 7.4) followed by dark incubation at 22 °C for 12–18 h. The cell

aggregates were centrifuged and washed two to three times with distilled water and then treated with 95% ethanol at 60 °C for 30 min. After being left stationary for 5 min, the absorbance was recorded at 485 nm with three replicates, and the boiled calli were used as the control. The viability assessed by TTC method (V_{TTC}) was calculated by the following formula: $V_{TTC} = T/C^* 100\%$, where T is the absorbance value of the treatment group, and C is the absorbance value of the control group.

2.4 Evans blue method

To determine changes in viability of cells by cold treatment, excised leaves were incubated with 4 mL of 0.25% aqueous Evans blue for 24 h and then washed with deionized water, until no more blue stain was eluted. The Evans blue incorporated into the leaves was extracted with 4 mL 0.1% sodium dodecyl sulfate (SDS). The eluent was diluted with four volumes of water, and the absorbance was determined at 600 nm. Boiled leaves that were completely dyed with Evans blue were used as the control (Baker and Mock, 1994). The viability assessed by Evans blue method (V_{EB}) was calculated by the following formula: $V_{EB} = [1 - (T/C)]^*100\%$.

2.5 In vitro selection of the embryogenic calli and regenerated plantlets

In vitro cultures, including the embryogenic calli and regenerated plantlets, that had undergone several generations of proliferation, were inoculated on the pre-cooled solid multiplication medium and subsequently treated at 0 °C for 72 h in growth chambers with a 16 h photoperiod. After treatment, cultures were moved to normal condition at 25 ± 1 °C. After 35 to 40 days of incubation, the surviving cultures were selected, transferred to solid multiplication medium, and sub-cultured every 30 days. The proliferated surviving cultures obtained from the first round of selection were transferred to the callus induction medium for 40 days of incubation and subsequently transferred to somatic embryo induction medium for 20 days of incubation. After five generations of subculture, all the obtained cultures continued to be treated at 0 °C for 72 h. The surviving cultures were selected and subcultured every 30 days. The same processing procedures were performed for the proliferated surviving cultures obtained from the previous round of selection. The embryogenic calli that survived after three rounds of selection were further cultured until the regeneration of intact plants.

2.6 PCR amplification and molecular marker analysis

The leaves of the obtained regenerated plants were collected and used for the DNA extractions, which were performed according to the protocol described by Ma et al. (2011). The vegetative progeny of the maternal plants were used as control. The quality of the extracted DNA was assessed with a NanoDrop 1000 spectrophotometer. The samples were then diluted to a working concentration of 10 ng μL^{-1}. The DNA was amplified using 26 ISSR primers from previously published reports (Silva et al. 2016; Vanijajiva 2012) (Supplementary Table 1). The reactions were performed with the following cycling profile: 94 °C for 3 min, followed by 40 cycles of 94 °C/45 s, 48 °C/45 s, 72 °C/1 min, and a final step of 7 min at 72 °C. The amplification products were separated on 2.5% agarose gel and photographed under UV light. Only the clear polymorphic fragments from the DNA amplification were considered, and the bands were counted according to the presence or absence in the gel.

2.7 Cytological analysis

Ploidy analysis was conducted by flow cytometer using Cystain DNA 2 STEP (Partec, Munster, Germany) according to Wang et al. (2016). Approximately 1 cm^2 of leaf was chopped in 0.5 mL of Partec HR-A buffer. After being filtered, the samples were stained with 1 mL of HR-B buffer. For each sample, at least 3000 cells were analyzed. Stomatal distribution was studied using nail varnish replicas as described by Tricker et al. (2005).

2.8 Determination of proline content and SOD activity

The protocol for determination of proline content was as published previously (Bates et al. 1973). The leaves was homogenized in 3% (w/v) aqueous sulfosalicylic acid and then centrifuged. The filtered homogenate was reacted with acidic-ninhydrin and acetic acid at 100 °C for 1 h. Toluene was then added to the cooled reaction mixture and mixed vigorously. The absorbance of filtrate at 520 nm was recorded using toluene as blank. Proline content was expressed as $\mu g\ g^{-1}$ FW. Superoxide dismutase (SOD) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), according to the method of Giannopolitis and Ries (1977).

2.9 Data analysis

Statistical analysis was performed using analysis of variance (ANOVA), and means and standard errors (SE) were used throughout. The data were analyzed using SAS V8.02, and differences between mean values were assessed by Duncan's multiple range test. A probability of $p < 0.05$ was considered significant.

3 Results

3.1 Establishment of the in vitro culture system

The callus tissues were induced from slips of the maternal plant. After more than 30 generations of multiplication and subculture, large amounts of in vitro cultures, including non-embryogenic calli, embryogenic cell suspensions, embryogenic calli, adventitious shoots (organogenesis), and regenerated plantlets (somatic embryogenesis), were obtained using the established in vitro culture system. Embryogenic calli and regenerated plantlets were the principal cultures used in the following experiments.

3.2 Evaluation of cold tolerance for the in vitro cultures

The above in vitro cultures were treated at five different temperature gradients for 6, 12, 24, 48, and 72 h, respectively. Cold tolerance was evaluated using individual survival rate and relative cell viability. As shown in Table 1, the individual survival rate and relative cell viability were significantly decreased along with the increase of the treatment time at 5 °C. All of the tested cultures, including embryogenic calli or regenerate plantlets, were completely dead at 0 °C for 24 h or longer and at −2 °C for 6 h or longer (Table 2).

As shown in Fig. 1, the red TTC-stained sections of embryogenic calli and the Evans blue–stained sections of regenerated plantlets became shallow or deepened gradually with the prolonged treatment time at 0 °C. Further detailed examination showed that the relative cell viabilities were gradually decreased along with the increase in cold stress intensity (Supplementary Fig. 1). To obtain somaclonal variants with stronger cold-tolerant abilities, more stringent treatment combination of 0 °C for 72 h was considered the lethal condition in the following selection experiments.

Table 1 Determination of the relative cell viability and individual survival rate of the in vitro cultures under different treatments

Treatment	Temperature (°C)	Time (h)	Relative cell viability (%)				Individual survival rate (%)			
			Embryogenic Calli		Regenerated plantlets		Embryogenic Calli		Regenerated plantlets	
1	8	6	95.63	a	95.08	a	96.96	a	96.88	a
2	8	12	90.72	ab	79.77	ab	90.32	ab	83.87	ab
3	8	24	79.61	b	76.84	b	80.00	b	80.00	b
4	8	48	70.23	bc	64.81	bc	77.14	b	70.00	b
5	8	72	60.45	c	57.51	c	64.51	c	58.06	c
6	5	6	88.27	a	85.26	a	89.65	ab	86.55	ab
7	5	12	70.96	c	70.88	bc	70.00	bc	73.33	bc
8	5	24	54.98	c	58.95	c	57.57	c	58.78	c
9	5	48	50.16	cd	45.43	cd	51.61	cd	51.61	cd
10	5	72	32.24	de	24.07	de	32.25	de	29.03	de
11	2	6	58.82	c	59.72	c	60.00	c	60.00	c
12	2	12	51.38	cd	28.31	cd	53.33	cd	30.00	cd
13	2	24	34.72	d	23.63	d	36.11	d	26.66	d
14	2	48	18.93	de	14.83	de	20.00	de	20.00	de
15	2	72	1.86	g	1.63	g	2.86	g	2.94	g
16	0	6	36.28	d	23.35	d	36.00	d	28.12	d
17	0	12	11.72	e	10.12	e	14.70	e	18.52	e
18	0	24	0.33	f	0.56	f	0.00	f	0.00	f
19	0	48	0.08	f	0.14	f	0.00	f	0.00	f
20	0	72	0.32	f	0.45	f	0.00	f	0.00	f
21	− 2	6	0.94	f	0.11	f	0.00	f	0.00	f
22	− 2	12	0.11	f	0.09	f	0.00	f	0.00	f
23	− 2	24	0.55	f	0.08	f	0.00	f	0.00	f
24	− 2	48	0.04	f	0.01	f	0.00	f	0.00	f
25	− 2	72	0.09	f	0.08	f	0.00	f	0.00	f

Table 2 The survival rate of the in vitro cultures at different periods of the cold selection

	Selection condition	Before selection		After selection		Survival rate (%)	
		Number of somatic embryos	Number of regenerated plantlets	Number of somatic embryos	Number of regenerated plantlets	Somatic embryos	Regenerated plantlets
1st round	0 °C + 72 h	2.33 × 106	4709	0	13	0	0.28
2nd round	0 °C + 72 h	6447	125	297	9	4.61	7.2
3rd round	0 °C + 96 h	771	67	28	0	3.63	0

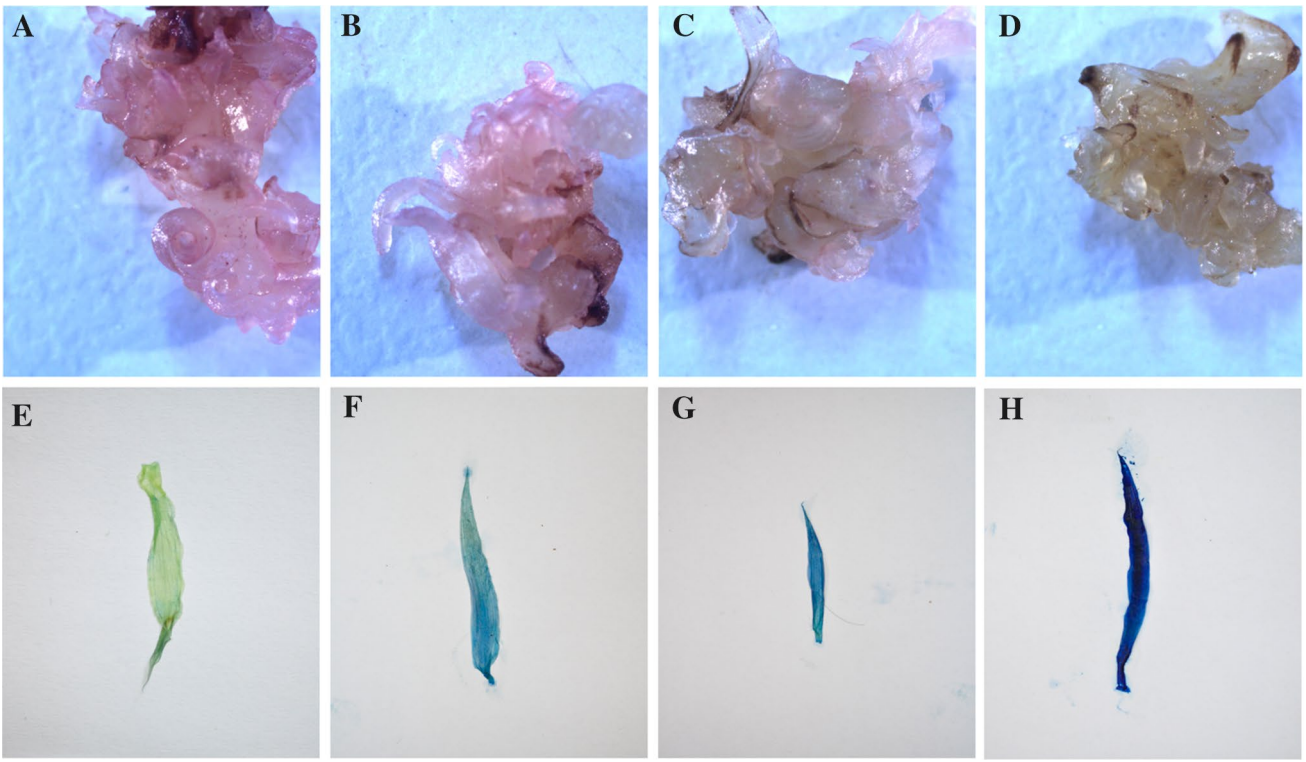

Fig. 1 Staining of the embryogenic calli and regenerated plantlets after cold treatment. **a-d** Staining of the embryogenic calli with the TTC method at 0, 4, 24, and 48 h after cold treatment, respectively. **e–h** Staining of the regenerated plantlets with Evans blue at 0, 4, 24, and 48 h after cold treatment, respectively

3.3 Three consecutive rounds of low-temperature selection for in vitro cultures

In vitro cultures derived from cold-tolerant plants, including about 6.29×10^4 g embryogenic calli (containing about 2.33×10^6 somatic embryos) and 4709 regenerated plantlets, were first treated at 0 °C for 72 h. The green surface of the treated embryogenic calli gradually became lighter and softer during the recovery phase (Fig. 2a–c). For almost all of the regenerated plantlets, the leaf chlorosis symptoms gradually worsened, apoptosis occurred (Fig. 2d–f), and the plantlets were completely dead (Fig. 2g, h) after treatments; only 13 surviving individuals were obtained (Fig. 2i–k), and no surviving embryogenic calli were found. Through five generations of multiplication and subculture, about 6447 somatic embryos and 125 regenerated plantlets from the 13 original surviving plantlets were obtained.

The above cultures underwent a second round of selection, and 297 somatic embryos and 9 plantlets survived at rates of 4.61% and 7.2%, respectively. The survival characteristics, including somatic embyogenesis and shoot regeneration, were observed in the interior growth point or exterior of the leaf base (Fig. 3a–d), while other old leaves showed a gradual chlorosis, browning, and wilting.

In the third round selection, the treatment time at 0 °C was extended to 96 h. All regenerated plantlets were completely dead, and almost all treated embryogenic calli exhibited surface browning (Fig. 3e). The formation of the single somatic

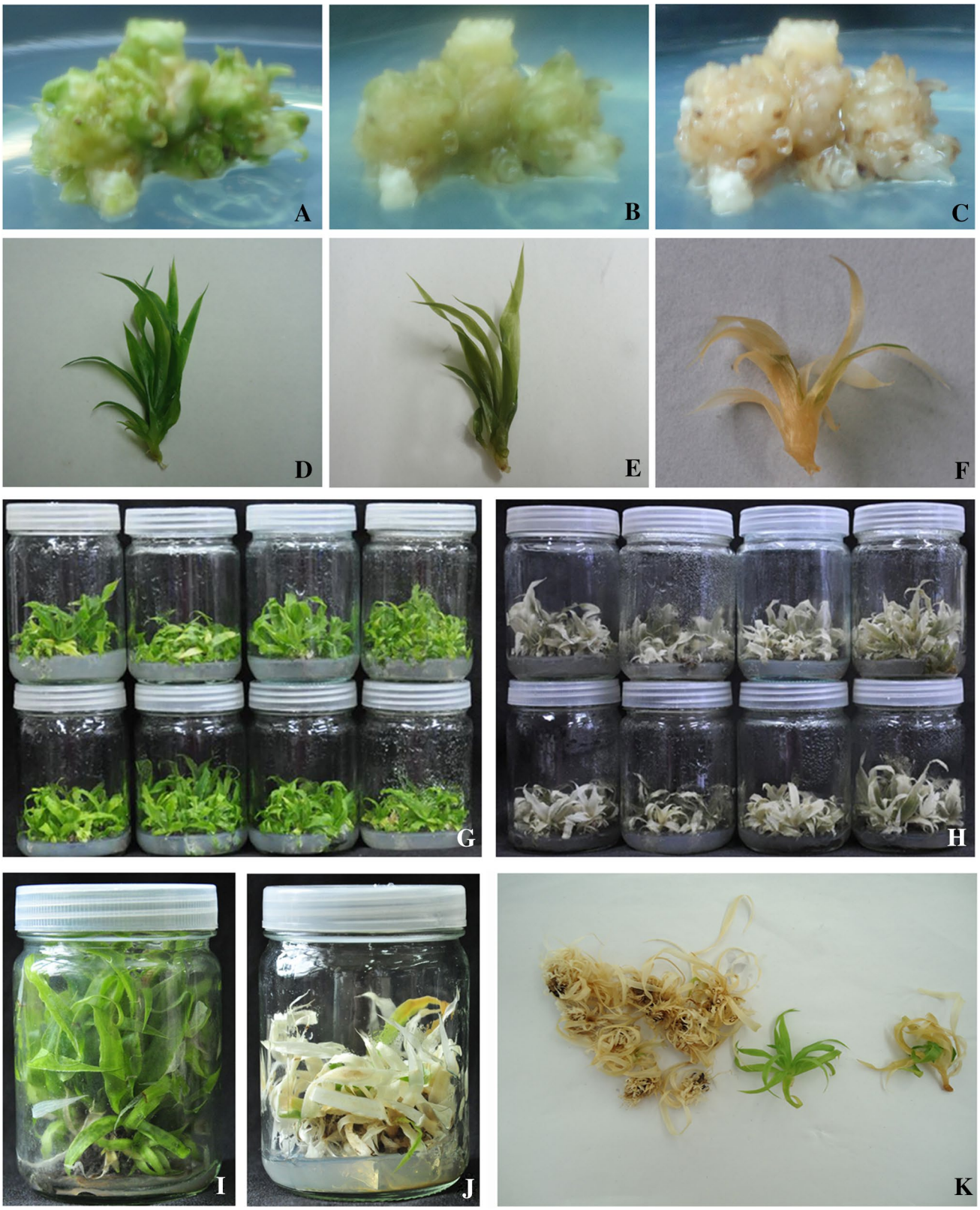

Fig. 2 In vitro cultures during the first round of low-temperature selection. The morphology of the embryogenic calli (**a-c**) and regenerated plantlets (**d-f**) during cold treatments. **g-h**: Morphology of the culture population before and after cold selection, respectively. **i-k** The surviving regenerated plantlets after the first round of selection

embryo was observed under a stereomicroscope at 35 days after transfer into the somatic embryo induction medium (Fig. 3f). The embryogenic cell mass was then developed and proliferated during the subsequent culture (Fig. 3g, h). A total of 67 regenerated plantlets and 771 somatic embryos were treated in this round of selection; 28 surviving embryogenic cell aggregates were obtained, and the survival rate of the somatic embryos was 3.63%. The plants regenerated from the survived embryos that underwent three rounds of cold selection could be regarded as clones from the same cold-selected line.

3.4 Molecular and cytological characterization of the cold-selected line

ISSR analysis was used to determine whether the genetic variations occurred in the cold-selected line compared to the control. As shown in Fig. 4a, the amplification profile of Primer ISSR15 showed polymorphic bands in six randomly selected individuals from cold-selected lines compared to the two individuals from control, and the occurrence of the somaclonal variations was confirmed. Therefore, the cold-selected line can be referred to as the cold-tolerant variant, and the variations may be correlated to the cold tolerance characteristics. It is noteworthy that no polymorphic differences were identified among individuals of the cold-tolerant variant, further demonstrating their uniform genetic composition and common origins.

The linear-fluorescence intensity histograms of relative nuclear DNA content of leaves from control and in vitro–selected cold-tolerant variant were analyzed using a flow cytometer. As shown in Fig. 4b, c, the relative nuclear DNA content was roughly the same between the control and the variant, and both were regarded as diploid, indicating that no major ploidy changes occurred during in vitro selection processes. No apparent morphological differences were found between the control and the variants. According to microscopy observations of the leaf abaxial surface, there were no significant changes in stomatal density (data not shown).

3.5 Cold tolerance evaluation of the cold-selected line

To evaluate cold tolerance ability, the plantlets from the cold-selected line and the control were subjected to 0 °C for 72 h. Compared to the complete chlorosis and death of the control, only a few was dead and most of the plantlets exhibited only mild chlorosis at 7 days after the cold treatments (Fig. 5a, b). The average survival rate of the cold-selected plantlets was up to 71.4%, indicating their significantly increased cold tolerance ability. The survival plantlets were continuously cultivated until the formation of the well-developed shoot and root systems, and then they were transplanted to pots with a mixture of loamy soil and sand. The well-grown potted plants were subjected to 0 °C for 72 h, and the cold-selected variant plants could maintain normal growth at 10 days after treatment (Fig. 5c). By contrast, the control plants were completely wilted and dead (Fig. 5d).

The proline content and SOD activity in variant and control plants were further analyzed. As shown in Fig. 5e, the proline contents were significantly increased at 72 h after

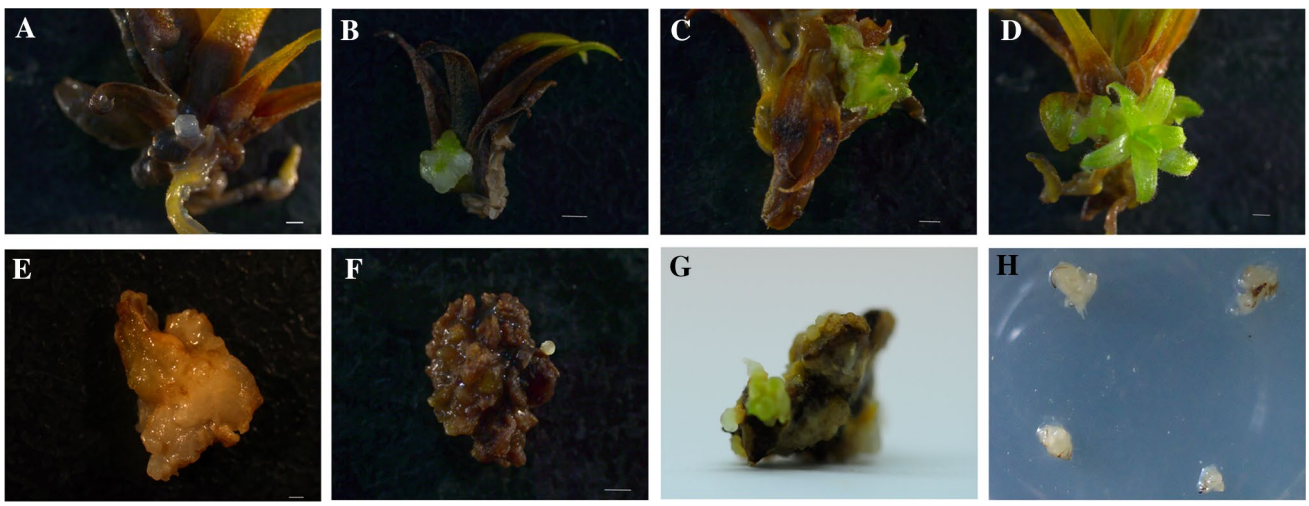

Fig. 3 In vitro cultures during the second and third rounds of cold selection. **a, b** The morphology of the surviving somatic embryos at the exterior of the leaf base of the cold-treated plantlets. **c, d** The morphology of the surviving regenerated plantlets at the exterior of the leaf base of the cold-treated plantlets. **e** The morphology of the embryogenic calli after the third round of cold selection. **f** The formation of the single somatic embryo on the surface of the cold-treated embryogenic calli. **g** The formation of the surviving embryogenic cell mass. **h** The proliferation of the surviving embryogenic cell mass. Scale bars = 1 mm

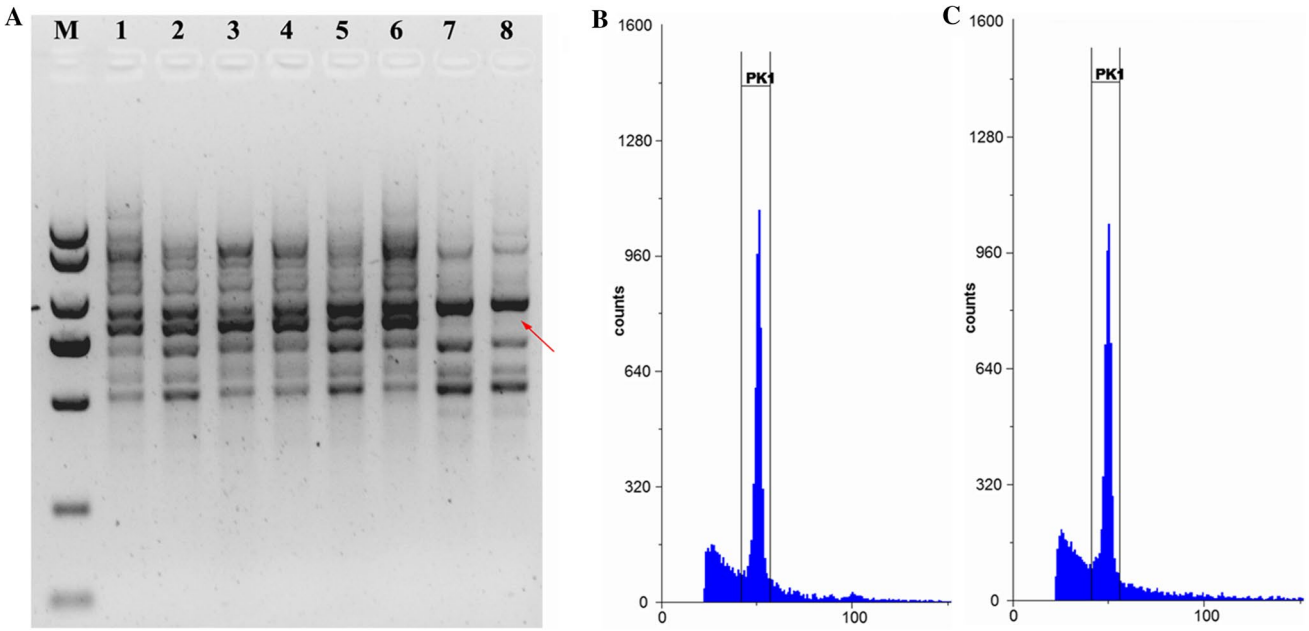

Fig. 4 The molecular marker and ploidy analysis for the regenerated cold-selected plants. **a** ISSR marker analysis for the regenerated cold-selected plants. M, marker. 1–6 indicate six randomly selected individuals from the cold-selected line. 7 and 8 indicate two individual control plants. The red arrows indicate the differential bands. **b** Ploidy analysis of the cold-selected line by flow cytometry. **c** Ploidy analysis of the control plant by flow cytometry

the cold treatments in both lines. It is noteworthy that the cold-induced increase in proline contents was more conspicuous in variant plants than in the control. Similarly, the cold-induced SOD activity was also observed in both lines, and the SOD activity after cold treatment was significantly greater in the variant than in the control (Fig. 5f). The higher survival rate and significantly increased SOD activity and proline accumulation in variant plants under cold stress indicated that the in vitro cold selection of somaclonal variations led to improved cold tolerance in the pineapple variant line.

4 Discussion

Somaclonal variation is of great practical interest due to its potential applications in plant breeding, and it is a promising strategy for the improvement of many vegetatively propagated crops such as banana (Hwang and Ko 2004), grapevine (Kuksova et al. 1997), sweet potato (Anwar et al. 2010), strawberry (Whitehouse et al. 2014), kiwi fruit (Caboni et al. 2003), pear (Nacheva et al. 2014), and peach (Hammerschlag 2000). Somaclonal variations have been previously characterized in pineapple, a typical vegetatively propagated herbaceous perennial, and attempts to improve it through this strategy have also been reported (Dhurve et al. 2021). Several of the pineapple variants were studied in detail and systemically identified. For example, the somaclonal variants Dwarf and P3R5 were different from the donor plant in several morphological characteristics and also exhibited different AFLP banding patterns (Pérez et al. 2012). Dhurve et al. (2021) found wide variability among 75 somaclonal variants of the pineapple cultivar 'Mauritius'. Kohpaii et al. (2017) investigated the cellular characteristics, biochemical specificities, and ISSR markers for the somaclonal variation in the in vitro–regenerated pineapple. Among these studies, several strategies, including phenotype evaluation, cytogenetic analysis, assessment of physiological and biochemical characteristics, and use of DNA-based molecular markers, were adopted in the assessment of the pineapple somaclonal variants.

In this study, a pineapple somaclonal variant with an increased cold tolerance was obtained. The phenotype variations between the variant and the control were mainly reflected by their different survival rates under lethal conditions of low temperature, which was also regarded as the most direct and effective evidence for the improved cold tolerance ability of the in vitro–selected variant. No significant variations were found at the cellular level, based on the ploidy level analysis with a flow cytometer and the microscopy observations. Variations in physiological and biochemical characteristics, as manifested by the significantly increased SOD activity and proline accumulation in variant plants under cold stress, were identified between the variant and the control. Moreover, the genetic alteration of the cold-tolerant variant was validated by ISSR analysis. Basing on the obtained phenotypic, cellular, physiological,

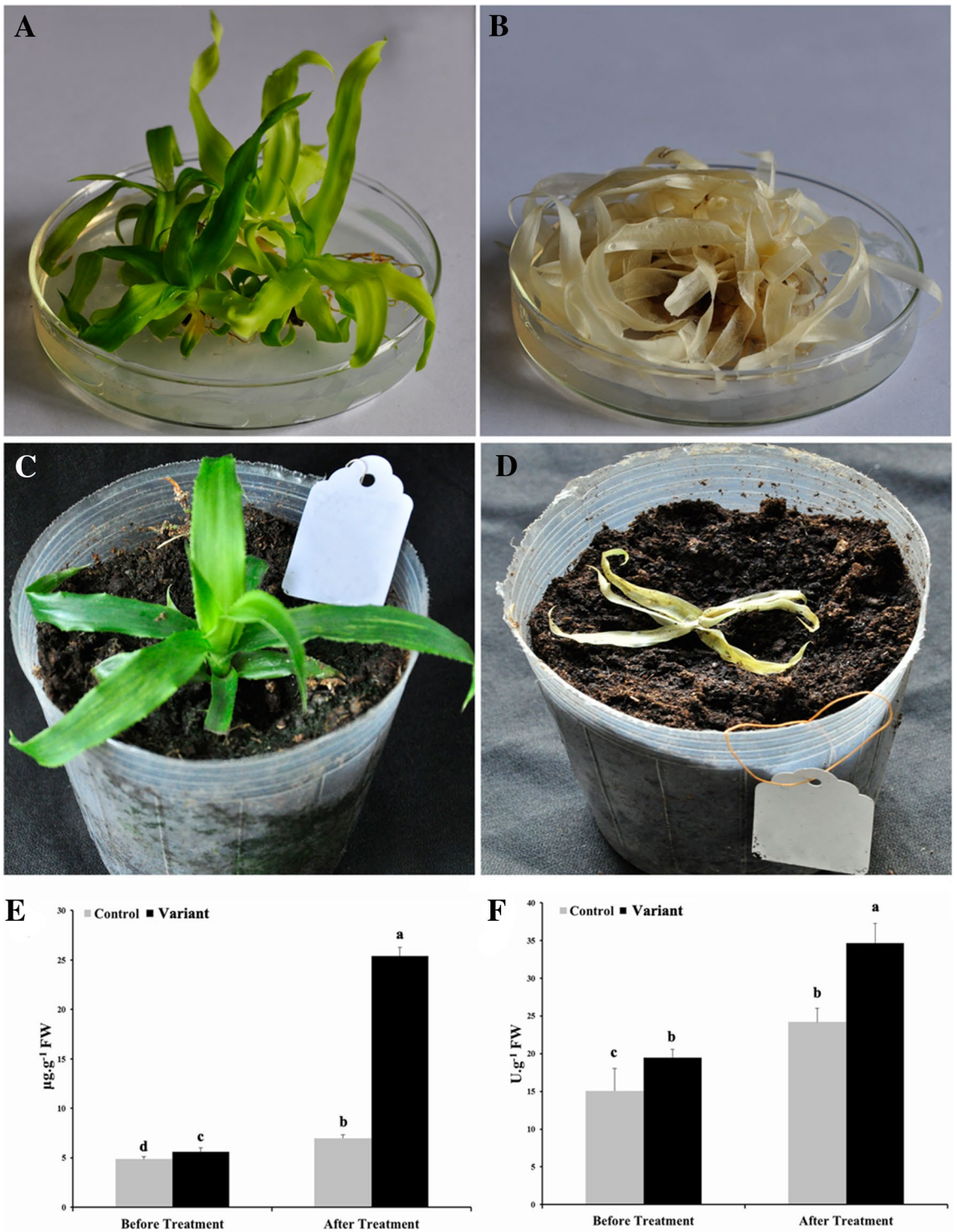

Fig. 5 Evaluation of cold tolerance in the cold-tolerant variant and control plants. **a, b** The morphology of the regenerated cold-tolerant variant and control plants at 7 days after cold treatment. **c, d** The morphology of the transplanted cold-tolerant mutant and control plants at 10 days after cold treatment. The proline contents (**e**) and SOD activities (**f**) in variant and control plants before and after cold treatment

biochemical, and molecular marker results, our findings revealed the existence of a somaclonal variation of cold-tolerant pineapple.

The somaclonal variations in the cold-tolerant variant might be due to the pre-existing spontaneous mutations. The donor pineapple plant used in this study, which survived

after a long period of cold, might possess spontaneous mutations related to cold tolerance. However, the mutations may be not stable, and using a traditional vegetative propagation method might lead to the loss of the cold-tolerant mutation trait. The tissue culture techniques can maintenance and enlarge the pre-existing variations in donor plants, and provided basis for the in vitro selections (Rai et al. 2011). In this study, a high-efficiency in vitro culture system was established for pineapple, whole-embryo culture techniques with minor modifications were adopted, and the embryogenic calli were used as the principal selecting objects. It was suggested that the origin of pineapple somatic embryos was unicellular (He et al. 2010; Ma et al. 2011); therefore, regeneration via embryogenesis could prevent the formation of chimera and possibly screen out the genetically uniform plantlets. The use of the pineapple in vitro cultures like embryogenic calli obtained in this study facilitated the separation of the mutations from the maternal plant and could provide a sufficient number of individuals for the following in vitro selection.

Moreover, the somaclonal variations in the cold-tolerant variant might also be generated de novo during the tissue culture under the influence of triggering conditions (Siddique, 2020). The tissue culture can induce variations in regenerated plants, and in vitro selection can considerably shorten the time for the selection of desirable traits under selection pressure with minimal environmental interaction (Anis and Ahmad 2016). In vitro selection for somaclonal variation in stress tolerance has been proven to be effective, and research about the selection of stress-tolerant variants have been widely reported (Rai et al. 2011). In pineapple, the dwarf pineapple variant with higher histone deacetylase enzyme activity was selected by Halim et al. (2018) through hormonal induction, NaCl, and abscisic acid supplementation. Although several pineapple somaclonal variants have been obtained and described in detail (Pérez et al. 2011), the cold stress–related variations and corresponding selections were not reported so far. In this study, an effective in vitro selection system for cold-tolerant variants of pineapple was developed, which could provide a reference for selecting other biotic or abiotic stress–tolerant pineapple variants.

The screening strategy was very important for the selection of the stress-tolerant variant, and different kinds of selecting agents, such as NaCl, mannitol, and pathogen culture, were applied to select and regenerate plants with desirable characteristics (Efferth 2019). In this study, extreme low temperature was used as the selecting agent, and the pineapple in vitro cultures were exposed to this stress for various periods of time, a similar strategy to one used in the selection of cold-tolerant variants in other species. For example, the seashore paspalum cold-tolerant mutant survived and was selected after two consecutive rounds of treatments with −7 °C for 24 h and −8 °C for 24 h, respectively (Liu et al. 2013). The chilling tolerance improvement for the embryo-derived calli of four rice varieties was obtained through different chilling treatments, such as 5 °C for 5 days or 10 °C for 9 days. It was also found that the chilling treatments need to be adapted according to the varietal sensitivity to low temperature, the higher the varietal tolerance, and the harder the chilling treatment (Bertin and Bouharmont, 1997).

The screening temperature and length of treatment were important components of the selective pressure in the in vitro cold selection. In this study, the cold-tolerant ability of the in vitro cultures derived from the donor pineapple plant was evaluated; 0 °C for 72 h was determined as the lethal condition and was adopted during the following cold selection. Two types of selection methods, including stepwise long-term treatment and shock treatment, were mainly used in the in vitro selection (Jain et al. 2013; Rai et al. 2011). For the shock treatment adopted in this study, the cultures were directly subjected to a shock of extreme temperature. In theory, only the in vitro cultures derived from the cold-tolerant-related mutant cells, which were pre-existing in the donor plant or induced during tissue culture, could survive and then be selected in the following procedures. The shock treatment could prevent the problem of epigenetic adaption during in vitro selection and may prevent the development of epigenetically adapted cells (Dix 1993; Tal 1994; Rai et al. 2011). The successive three rounds of shock treatments in our study could contribute to screening out more stable genetic variants. The selection strategy used in this study could be helpful to implement highly efficient screening of the pineapple cold-tolerant variant.

Based on the high-efficiency embryo culture system and the low-temperature stress selection strategy, the pineapple variant that exhibited significant improvement in cold tolerance was successfully screened out, as manifested by the higher survival rate and increased proline content and SOD activity compared with the control. Certainly, the field performance of the selected variant still needs to be evaluated and monitored at multiple planting sites and during several vegetative generations before concluding its value in pineapple breeding. The potential reasons for the cold-tolerant variant may be very complicated, it was found that the somaclonal variation may result from different causes like single-gene mutations, transposable element activation, and altered expression of multigene families. Although the genetic alterations of the pineapple variant have been validated at the DNA level with ISSR molecular markers, the markers may not actually be within a gene or even very close to one, and the band polymorphisms may not be associated with the observed cold tolerance differences. Up to now, research about the cold-tolerant mechanism in pineapple was rare. The previous pineapple transcriptome profiling under low-temperature stress revealed the global cold response patterns (Chen et al. 2016). The cold tolerance

was known to be under polygenic control, and the genes were scattered over the genome (Frascaroli 2018). It seems likely that the improvement of cold tolerance in variants could be due to a gross genetic change, and the application of new technology like next-generation sequencing might be helpful to better understand the genetic background and the cold-tolerant mechanism of the variant. Above all, the cold-tolerant variant obtained in this study could be used to explore the molecular and genetic mechanism associated with improved cold tolerance, which is of great value for the genetic improvement of pineapple cold tolerance.

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Declarations

Conflict of interest There is no conflict of interest between the authors.

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